

TIME-DEPENDENT CHANGES OF IMMUNOLOGIC RESPONSES AFTER BURN INJURY AND IMMUNOMODULATION BY CIMETIDINE AND PYRIMETHAMINE IN AN ANIMAL MODEL

JAFARZADEH A^{1*}, HASSAN ZM², EBTEKAR M², MOHAGHEGH-HAZRATI S³
AND TIRAIHI T⁴

¹Department of Immunology, Medical School, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

²Department of Immunology, Tarbiat Modarres University, Tehran, Iran

³Department of Immunology, Tehran University of Medical Sciences, Tehran, Iran

⁴Department of Pathology, Tarbiat Modarres University, Tehran, Iran

ABSTRACT

Severe suppression of the immune system is the major cause of infections following burn injury. The aim of this study was to investigate the time-related alterations of immune responses following thermal injury in an animal model and also to modulate immune responses by use of the immunomodulators cimetidine and pyrimethamine. Male Balb/c mice were anesthetized and given a 10% total body surface area full-thickness burn. The time-dependent changes of delayed type hypersensitivity (DTH) and antibody responses to sheep red blood cell (SRBC) were assessed at post-burn days (PBD). The effects of different doses of cimetidine and pyrimethamine on DTH response were also quantitated at 10 PBD.

Marked suppression of DTH response occurred during 30 days after burn trauma, with maximal suppression occurring between 10 to 14 days after burn injury. Simultaneously the antibody response to SRBC was significantly increased after thermal trauma. Cimetidine (at doses of 10 and 15 mg/kg) and pyrimethamine (at doses of 5 and 10 mg/kg) significantly augmented DTH response after thermal injury.

These results showed that the severe time-dependent alterations occurred in DTH and antibody responses following burn injury. Cimetidine and pyrimethamine also restore burn-induced suppression of DTH response following thermal trauma.

Keywords: Burn, delayed type hypersensitivity, antibody response, cimetidine; pyrimethamine, immunosuppression, immunomodulation, animal model.

INTRODUCTION

Thermal injury induces immune suppression which makes burned patients susceptible to infections (Church *et al.*, 2006). It has been reported that the effector mechanisms of the immune system such as phagocytosis, chemotaxis, lymphocyte proliferation and antibody production are impaired following thermal trauma (Kawakami *et al.*, 1998, Steintraesser *et al.*, 2004). Previous investigations have consistently showed the suppression of the cell-mediated immune responses at post-burn period and increased predisposition to succeeding septic complications and mortality (Schwacha *et al.*, 2002). Accordingly, the failure of T lymphocytes response is believed to be a prominent immunological consequence of thermal injury (Dalton, 2001). The burn-associated cell-mediated immunity suppression has been attributed largely to hyperproduction of inflammatory cytokines (O'Sullivan *et al.*, 1997), reduction in the percentages of T lymphocytes (Dong *et al.*, 2007), suppression of cytokine synthesis by T cells such as IL-2 and IFN- γ (Schwacha *et al.*, 2005), elevated levels of soluble IL-2 receptors (Correia *et al.*, 2002), an imbalance in Th1 and

Th2 functions and excessive production of Th2-type cytokines including IL-4 and IL-10 (Schwacha *et al.*, 2003), higher production of macrophage's inhibitory mediators such as prostaglandin E2 (Schwacha *et al.*, 2003), increase in the regulatory T cell activity (MacConmara *et al.*, 2006), elevation of levels of stress-associated hormones (Atiyeh *et al.*, 2001) and generation of a number of immunosuppressive peptides (Huang, 1992).

Some investigators also have assessed the relationship between burn injury and antibody response with controversial results, reporting unchanged (Shorr *et al.*, 1984), diminished (Kinoshita *et al.*, 2006) or even increased (Thomson *et al.*, 1991; Nijsten *et al.*, 1991) humoral immunity following thermal injury.

It has been reported that the burn-induced immunosuppression could be improved by methods including surgical excision of burn wound (Yamamoto *et al.*, 1996), use of antioxidants (Cetinkale *et al.*, 1999), use of immunomodulating drugs such as indomethacin (Choudhry *et al.*, 2002), cimetidine (Gharegozloo *et al.*,

*Corresponding author: e-mail: jafarzadeh14@yahoo.com

2004), ibuprofen (Freeman *et al.*, 1998), cerium nitrate (Sparkes *et al.*, 1993), cyclophosphamide (Waymack *et al.*, 1988), use of cytokines such as recombinant interleukin-2 (Gough *et al.*, 1988) and thymopeptin (Xin *et al.*, 2001). This study was conducted to evaluate the time-dependent alterations in cellular and antibody responses at post-burn period and also to evaluate the effects of the two immunomodulators cimetidine and pyrimethamine on immune responses using an animal model.

MATERIALS AND METHODS

Animals

Eight-week-old male Balb/c mice were purchased from Pasteur Institute, Tehran, Iran. They were given sterilized water and autoclaved standard mouse chow *ad libitum* throughout the study.

Mice and burn injury

The thermal injury protocol was performed according to previously described method (27, 28). The mice were anesthetized by i.p. administration of pentobarbital (1 mg/mouse). The dorsal furs of mice were shaved and the animals were held in a plastic mold so that 10% of the total body surface area (TBSA) at dorsal surface was exposed. The shaved exposed dorsum was then submerged in 90°C water for 9 s. We observed that this procedure histologically results in a burn injury with full-thickness degree. The unburned sham mice only had their backs shaved without any burn injury. The mortality rate of this burn injury varied from 0 to 5%. TBSA along the dorsum was calculated based on a formula for a 20 g mouse: $A=KW^{2/3}$, where A =area in cm^2 ; $K=8.95$; W =body weight in grams (Walker *et al.*, 1968, Alexander *et al.*, 2005).

Drug

- Cimetidine (Tagamet) was purchased from Chemidarou Company, Iran. Doses of 5, 10 and 15 mg/kg of this drug were administered daily by i.p. injection from day 0 up to day 10.
- Pyrimethamine (Daraprim) was obtained from Wellcome Company, England. Three doses of 2.5, 5 and 10 mg/kg were selected on the basis of the drug's effectiveness and toxicity. One single injection on day 0 was sufficient to produce the desired effects (Ebtekar *et al.*, 1993; Hassan *et al.*, 2001; 2002; Gharegozloo *et al.*, 2004). The drugs were injected 3-4 h after burn injury. All doses of drugs were administered in saline in a total volume of 0.1 ml.

Antigen

Sheep red blood cells (SRBC) were obtained from Pasteur Institute, Tehran, Iran and preserved in sterile Alsevers' solution. After 3 times of washing, the necessary suspension was prepared for priming the animals.

Detection of delayed type hypersensitivity (DTH) response

DTH responses were evaluated by priming the mice with 1×10^8 SRBC injected subcutaneously in the back on day 0. The sensitized animals were challenged with 1×10^8 SRBC injected subcutaneously on the left hind footpad on day 5. Foot thickness was measured 24 h later with a Mauser dial caliper and the results were expressed as percent of increased foot thickness (Ebtekar *et al.*, 1993; Hassan *et al.*, 2001; 2002; Gharegozloo *et al.*, 2004). The DTH response was assessed at 3, 6, 8, 10, 14, 20, 30 and 40 PBD.

Detection of antibody response

The antibody responses were assessed by priming mice with 1×10^9 SRBC injected intraperitoneally at day 0. The mice were then bled by intracardiac puncture on day 5 and the antibody responses were assessed. Two-fold dilutions of the sera were made in phosphate buffered saline with a total volume of 0.2 ml. Then 0.2 ml volumes of 3% v/v washed SRBC were added to 96-well Nunc microtitre plate. Plates were subsequently incubated at 37°C for one hour and observed for hemagglutination under an inverted microscope. Results were expressed as Log₂ titer (Ebtekar *et al.*, 1993, Hassan *et al.*, 2001; 2002). The antibody response was assessed at 3, 4, 6, 10, 14, 20 and 30 PBD.

Histological preparation of the spleen

Spleens (removed upon autopsy at 10 PBD) were preserved in 10% formalin and processed for histological work. Tissues were dehydrated and implanted in paraffin. The sections with 3 μ m thickness were cut and stained with hematoxyline and eosin.

Spleen weight and organ index: Since the spleen is one of the major lymphoid organs indicative of immune function, the animals' body weight and the weight of their spleens were recorded at 10 PBD and the weight index was calculated according to the following formula (Ebtekar *et al.*, 1993; Hassan *et al.*, 2001; 2002; Gharegozloo *et al.*, 2004):

$$\text{Spleen weight index} = \frac{\text{spleen weight}}{\text{animal weight}} \times 100$$

STATISTICAL ANALYSIS

Differences in variables were analyzed using ANOVA and *t*-test as appropriate and P-values of less than 0.05 were considered significant. All the available data were analyzed by a computer program (SPSS, Chicago, IL, USA).

RESULTS

The Effect of Burn Injury on DTH Response

The alterations in DTH response during 40 days of post-burn were demonstrated in fig. 1. The DTH response of

control group was 34.54 ± 3.3 . The DTH responses of burn-exposed groups were: 24.7 ± 0.8 at 3 PBD, 20.1 ± 5 at 6 PBD, 16 ± 2.9 at 8 PBD, 12.62 ± 0.5 at 10 PBD, 11.81 ± 0.93 at 14 PBD, 11.81 ± 4 at 20 PBD, 26.7 ± 2.16 at 30 PBD and 30.98 ± 1.57 at 40 PBD. At 3 PBD the DTH response was significantly reduced as compared to control group ($P < 0.005$). The suppression of DTH response progressively increased with advancing time of post-burn with maximal suppression occurring during 10 to 14 days after burn injury. Up to 30 PBD, the DTH response was significantly lower than that observed in control group ($P < 0.0001$). However, there was no significant difference between DTH response of control group and that in burned exposed animals at 40 PBD.

The effect of burn injury on antibody response

The alterations in antibody response during 30 days of post-burn were demonstrated in fig. 2. The mean titer of anti-SRBC response in control group was 4.2 ± 0.44 . The mean titer of antibody responses in burn-exposed groups were: 8.6 ± 0.89 at 3 PBD, 9 ± 1.22 at 4 PBD, 11 ± 0 at 6 PBD, 8.8 ± 0.44 at 10 PBD, 7.5 ± 1.22 at 14 PBD, 6.8 ± 1.3 at 18 PBD, 5.4 ± 0.45 at 24 PBD and 5.25 ± 0.5 at 30 PBD. At 3 PBD the mean titer of anti-SRBC response was significantly higher as compared to control group ($P < 0.0001$). The antibody response progressively increased with advancing time of post-burn up to 6 PBD and then progressively decreased. Up to 30 PBD the antibody response was significantly higher than that observed in control group ($P < 0.05$).

The effects of cimetidine on burn-induced DTH suppression

The effect of the immunomodulator cimetidine on burn-induced DTH suppression is provided in table 1. As

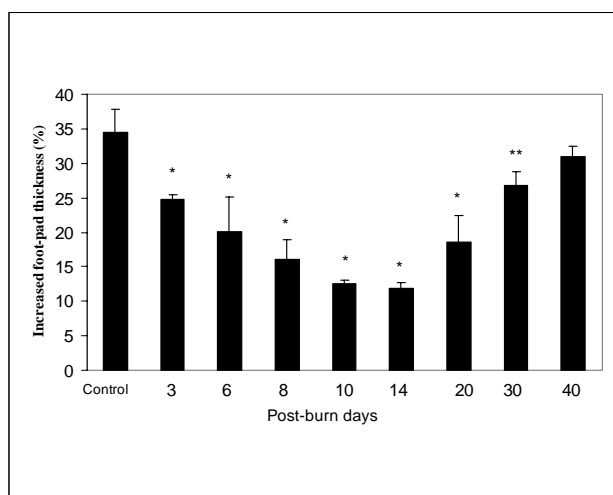


Fig. 1: Time-dependent alterations of delayed type hypersensitivity response at post burn. Up to 30 PBD the DTH response was significantly lower than that observed in control group (* $P < 0.0001$, ** $P < 0.005$).

shown in table 1, in order to assess the effect of cimetidine on the DTH response, mice were divided into 5 groups. Groups 1-3 were exposed to burn injury and received 5, 10 and 15 mg/kg doses of cimetidine, respectively. Group 4 was exposed to thermal injury without any drug administration (positive control group). Group 5 was not exposed to thermal injury and received no drug (negative control group). We observed that cimetidine at doses of 10 and 15 mg/kg significantly augmented DTH response as compared to positive control group ($P < 0.005$). However, the difference in DTH response of 5 mg/kg group and positive control group was not statistically significant. Moreover, there was no significant difference between the DTH response of 10 and 15 mg/kg doses as compared to negative control group. Furthermore, the difference in DTH response between 10 and 15 mg/kg doses was not significant.

The effects of pyrimethamine on burn-induced DTH suppression

Similarly as shown in Table 2, in order to assess the effect of pyrimethamine on the DTH response, mice were divided into 5 groups. Groups 1-3 were exposed to burn injury and received 2.5, 5 and 10 mg/kg doses of pyrimethamine, respectively. Group 4 was exposed to thermal injury without any drug administration (positive control group), while group 5 was neither exposed to thermal injury nor any drug was administered (negative control group). We observed that pyrimethamine at doses of 5 and 10 mg/kg significantly augmented DTH response as compared to positive control group ($P < 0.01$). However, the difference between DTH response of 2.5 mg/kg group and positive control group was not statistically significant. Moreover, there was no significant difference between DTH response of 5 and 10 mg/kg groups and also

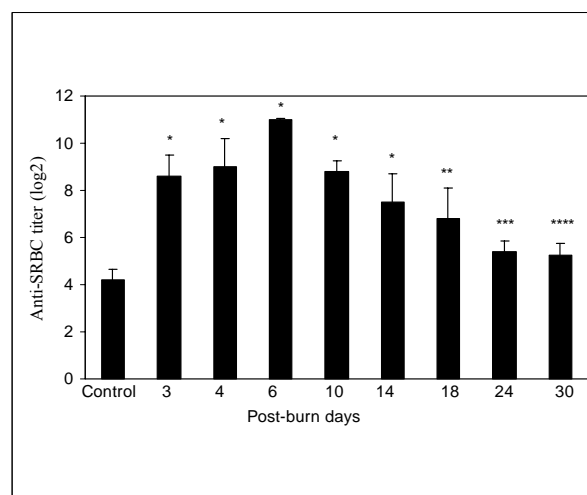


Fig. 2: Time-dependent alterations of antibody response at post burn. Up to 30 PBD the titer of anti-SRBC antibodies was significantly higher than that observed in control group (* $P < 0.0001$, ** $P < 0.005$, *** $P < 0.01$, **** $P < 0.05$).

between both groups as compared to negative control group.

The effects of immunomodulators on the spleen parameters

The effects of immunomodulators on the spleen histology and spleen weight index are also demonstrated in tables 1 and 2. Burn injury caused low-grade hyperplasia and enlarged follicles in the spleen; however, it induced no significant changes in spleen weight index. Histologically, cimetidine (at doses 10 and 15 mg/kg) and pyrimethamine (at doses 5 and 10 mg/kg) augmented hyperplasia in the spleen parenchyma, but had no significant effects on the spleen weight index.

DISCUSSION

The results of the present study demonstrated the marked suppression of DTH responses during 30 days after burn trauma, with maximal suppression occurring between 10 to 14 days after burn injury. Some investigators have studied post-burn time-dependent changes of immunological parameters in burned patients and also in animal models. In a study concerning the immunological changes of T lymphocyte in severely burned patients with sepsis, it has been reported that the proliferation of T lymphocytes and the levels of IL-2 production were significantly decreased in patients on 1, 14, 21, and 28 PBD. The

percentage of CD3+/CD4+ T lymphocytes were also decreased on 1, 5, 14, 21, 28 PBD (Dong *et al.*, 2007). Liu *et al.* have studied the changes in the percentage of CD14⁺ monocytes expressing HLA-DR in burned patients. They showed that the percentage of monocytes was significantly reduced on 3 PBD. The counts of monocytes in moderately burned patients partially recovered on 21 PBD while in severely burned patients it continued to be low till being discharged from the hospital. There were also significant differences in counts of monocytes between severely and moderately burned patients on 3, 7 and 14 PBD (Liu *et al.*, 2003). Furthermore, Hunt *et al.* demonstrated that cytotoxic T lymphocytes (CTL) response in burned rats decreased significantly at 3 PBD and returned to baseline in 7-10 PBD (Hunt *et al.*, 1998). Organ *et al.* have reported that after severe thermal injury in the rat, significant lymphopenia was observed in the peripheral blood along with depletion of lymphocytes from the spleen and thymus at 2 PBD. Lymphocytes in the bone marrow and cervical lymph nodes also decreased significantly while their numbers in the spleen and thymus remained depressed at 6 PBD. At day 60 after injury, lymphocyte numbers in all tissues were normalized (Organ *et al.*, 1989).

A relationship between burn, elevated IL-6 production, and diminished DTH response has been described.

Table 1: Effect of cimetidine on immune parameters following burn injury

Groups	Cimetidine (mg/kg)	Spleen index	Spleen Histology	% Footpad Increased (DTH)
1	5	0.56 ± 0.14	Low grade hyperplasia, enlarged follicles	9.75 ± 3.1
2	10	0.78 ± 0.21	Hyperplasia, enlarged follicles	23.41 ± 4.5*
3	15	0.61 ± 0.04	Hyperplasia, enlarged follicles	21.88 ± 3.36*
4	Control (+)	0.59 ± 0.05	Low grade hyperplasia, enlarged follicles	9.74 ± 1.7
5	Control (-)	0.65 ± 0.06	Normal histology	27.22 ± 3.36

*Represents that the DTH responses were significantly higher in comparison to positive control group (P<0.005).

Table 2: Effect of pyrimethamine on immune parameters following burn injury

Groups	Pyrimethamine (mg/kg)	Spleen index	Spleen Histology	% Footpad Increased (DTH)
1	2.5	0.78 ± 0.09	Hyperplasia, enlarged follicles	7.86 ± 0.45
2	5	.89 ± 0.13	Hyperplasia, enlarged follicles	13.84 ± 0.45*
3	10	0.91 ± .28	Hyperplasia, enlarged follicles	13.51 ± 2.81*
4	Control (+)	0.88 ± 0.38	Low grade hyperplasia, enlarged follicles	5.66 ± 1.82
5	Control (-)	0.68 ± 0.13	Normal histology	16.09 ± 2.47

*Represents that the DTH responses were significantly higher in comparison to positive control group (P<0.01).

Interestingly, it has been reported that the production of IL-6 by stimulated macrophages is significantly elevated at 10 PBD (Durbin *et al.*, 2002). Additionally, TGF- β has immunosuppressive activity, which suppresses immune responses (Meert *et al.*, 1996). With regard to burn injury, it has been demonstrated that plasma concentration of TGF- β was elevated at 6-8 days post-injury (Varedi *et al.*, 2001). PGE2 is also known to suppresses T cell proliferation and IL-2 production in many inflammatory conditions. It has also been shown that capacity of macrophages to produce PGE2 significantly increases after thermal injury and the suppression of T cell proliferation and IL-2 production at 3 PBD is a PGE2-mediated process (Schwacha *et al.*, 2003). In our study, the time course of the alteration in DTH response is closely similar to post-burn immunosuppression observed by other investigators.

We have also demonstrated that in parallel to diminished DTH responses, the antibody response to SRBC was simultaneously increased after thermal trauma. Some investigators have assessed the relationship between burn and antibody responses, reporting unchanged (Shorr *et al.*, 1984), diminished (Kinoshita *et al.*, 2006) or even augmented responses (Thomson *et al.*, 1991, Nijsten *et al.*, 1991). The possible mechanism by which thermal injury may influence antibody response remains to be known. However, the imbalances in Th cell function may have an important role. Th cells have been categorized with regard to their cytokine profiles. Th1 cells secrete cytokines such as IL-2 and IFN- γ which are responsible for cell-mediated immunity whereas Th2 cytokines (such as IL-4, IL-5, IL-6 and IL-10) support antibody responses (Skapenko *et al.*, 2007). It should be noted that IL-12 which is produced by macrophages and dendritic cells has potent properties for induction of Th1 responses (Skapenko *et al.*, 2007). Previous studies have demonstrated that thermal injury diminishes the IL-12 production and the Th2 cytokine IL-4 is also responsible for the inhibition of IL-12 production at post-burn (Utsunomiya *et al.*, 2001). Since Th1 and Th2 cytokines influence each other in a negative manner, a possible explanation for augmentation of antibody responses following burn trauma may be due to T cells differentiation towards the expression of a Th2 response which supports and suppresses humoral and cellular immune responses, respectively.

We have also investigated the ability of pyrimethamine and cimetidine to reverse immunosuppression induced by thermal injury. Our results demonstrated that cimetidine (at doses 10 and 15 mg/kg) and pyrimethamine (at doses 2.5 and 5 mg/kg) significantly increased DTH response following burn injury. Pyrimethamine is a 2,4-diaminopyrimidine developed almost fifty years ago for the treatment of malaria (Falco *et al.*, 1951). Its major mechanism seems to be the inhibition of the dihydrofolate

reductase enzyme, which is necessary for the biosynthesis of purines and pyrimidines (Leslie *et al.*, 1985). Thong *et al.* had shown that pyrimethamine administered to mice could stimulate both humoral and cell-mediated immunity against SRBC (Thong *et al.*, 1980). Moreover, it has been demonstrated that pyrimethamine at the dose similar to that we employed can improve burn blister fluid and sulfur mustard-induced immunosuppression (Ebtekar *et al.*, 1993; Schwacha *et al.*, 2003).

Cimetidine is a histamine (H₂) antagonist widely used for the treatment of duodenal ulcers and other gastric hypersecretory conditions (Kumar 1990). It has been shown that cimetidine can reverse histamine-induced suppression of the immune response. Cimetidine has also been implicated in augmentation of cell-mediated cytotoxicity and in the abrogation of suppressor T-cell function (Scheinfeld, 2003). Cimetidine has been effective in countering the burn wound itch (Baker *et al.*, 2001) tumor immunotherapy, as well as certain degrees of protection against infection in experimental animals (Ishikura *et al.*, 1999; Takahashi *et al.*, 2002). Cimetidine, at the dose similar to that employed in our study, has been proved to abrogate the burn blister fluid and sulfur mustard-induced immunosuppression (Ebtekar *et al.*, 1993; Schwacha *et al.*, 2003).

It should be noted that our study has several limitations which should be considered in future investigations. The first and foremost limitation is that an animal model may not be extended to the immunosuppression or immunomodulation as occurring in human. Second, measurement of cytokines especially Th1- and Th2- type cytokines were not part of the protocol. However, better understanding of the pro-inflammatory and anti-inflammatory responses to burn injury may allow the development of appropriate therapeutic strategies to improve outcome. In the current study, only male animals were used. Recent studies have shown that thermal injury differentially influences the immune system of male and female animals (Plackett *et al.*, 2010). Accordingly, the exact role of gender and sex hormones in response to thermal injury and immunomodulation in both genders requires further studies.

In conclusion, these results showed that the severe time-dependent alterations occurred in DTH and antibody responses following burn injury. Cimetidine and pyrimethamine also restore burn-induced suppression of DTH response following thermal trauma. Moreover, this study presents an ideal rodent model of burn exposure which can be used for further investigations in this area.

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